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1) FEBS Lett 1996 May 27;387(1):7-10
Reoxidation of the NADPH produced by the pentose phosphate pathway is necessary for the utilization of glucose by Kluyveromyces lactis rag2 mutants.
Gonzalez Siso MI, Freire Picos MA, Cerdan ME

2) Microbiology 1995 Feb;141 (Pt 2):385-91
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Dickinson JR, Sobanski MA, Hewlins MJ

3) J Bacteriol 1997 Apr;179(8):2724-30
Interruption of the phosphoglucose isomerase gene results in glucose auxotrophy in Mycobacterium smegmatis.
Tuckman D, Donnelly RJ, Zhao FX, Jacobs WR, Connell ND

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Mascarenhas D, Ashworth DJ, Chen CS

Thank you,
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In *Saccharomyces cerevisia* deletion of phosphoglucose isomerase can be suppressed by increased activities of enzymes of the hexose monophosphate pathway

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***Saccharomyces cerevisiae* mutants defective in the structural gene *PGI1* lack phosphoglucose isomerase and hence cannot grow on glucose. Spontaneous mutants were isolated by selecting for the regained ability to grow on YEPD (yeast extract/peptone/glucose). Three complementation groups called *spg29–31* (suppressor of *pgi1Δ*) were identified. The metabolism of [2-^{13}C]glucose was studied by ^{13}C NMR spectroscopy. This led to the conclusion that in a *spg29* mutant suppression of the glycolytic defect was achieved by increased carbon flux through the hexose monophosphate pathway. The specific activities of enzymes of the hexose monophosphate pathway (except glucose-6-phosphate dehydrogenase) and NAD- and NADP-dependent glutamate dehydrogenase were increased in the bypass mutant.**

Keywords: *Saccharomyces cerevisiae*, *pgi1Δ* suppressor mutations, hexose monophosphate pathway, ^{13}C NMR

INTRODUCTION

Phosphoglucose isomerase is the enzyme which catalyses the interconversion of glucose 6-phosphate and fructose 6-phosphate. Thus, it is both the second step of the glycolytic sequence and the last step of gluconeogenesis. Mutants of *Saccharomyces cerevisiae* defective in the structural gene (*PGI1*) for phosphoglucose isomerase have been described by many authors (Maitra, 1971; Herrera & Pascual, 1978; Clifton *et al.*, 1978; Ciriacy & Breitenbach, 1979; Aguilera, 1986). All such *pgi1* mutants cannot grow on glucose, the assumption being that in *S. cerevisiae* flux through the hexose monophosphate pathway is inadequate to support growth. It is reckoned that the hexose monophosphate pathway is only capable of 8% of the carbon flux that occurs during growth on glucose (Bruinenberg *et al.*, 1986). In this respect *S. cerevisiae* is different from both *Escherichia coli* and *Kluyveromyces lactis*, in which mutants lacking phosphoglucose isomerase can still grow on glucose (Vinopal *et al.*, 1975; Goffrini *et al.*, 1991).

One way of trying to understand all aspects of the phenotype of *pgi1* mutants has been the isolation of extragenic suppressor mutations which confer upon *pgi1* mutants the ability to grow on glucose. Aguilera (1987) described *spg1* (suppressor of *pgi1Δ*) mutations which restored growth on glucose and resulted in obligately high levels of mitochondrial respiration and no ethanol formation. Gamo *et al.* (1993) described *rgl1* and *rgl2* (resistance to glucose) mutations from which it was also presumed that glucose may be channelled through the hexose monophosphate pathway to respiration. Recently, Boles *et al.* (1993) showed that overexpression of *GDH2*, which encodes the NAD-dependent glutamate dehydrogenase, can suppress the growth defect on glucose caused by *pgi1Δ* mutations. The explanation for this is that overexpression of NAD-dependent glutamate dehydrogenase causes a cycle of metabolic interconversion between 2-oxoglutarate and glutamate in which the anabolic reaction results in the conversion of 2-oxoglutarate to glutamate with concomitant conversion of NADPH to NADP, whilst the catabolic reaction results in the formation of 2-oxoglutarate from glutamate with conversion of NAD to NADH. Hence, the rapid depletion of NADP which would otherwise result from overuse of glucose-6-phosphate dehydrogenase (if flux via the hexose monophosphate pathway was increased in *pgi1Δ* bypass mutants) is avoided and the extra NADH which is formed can be oxidized by the electron transport chain. This also explains the high obligatory respiration observed in *pgi1* bypass mutants.

Boles *et al.* (1993) also showed that deletion of the *ZWF1*

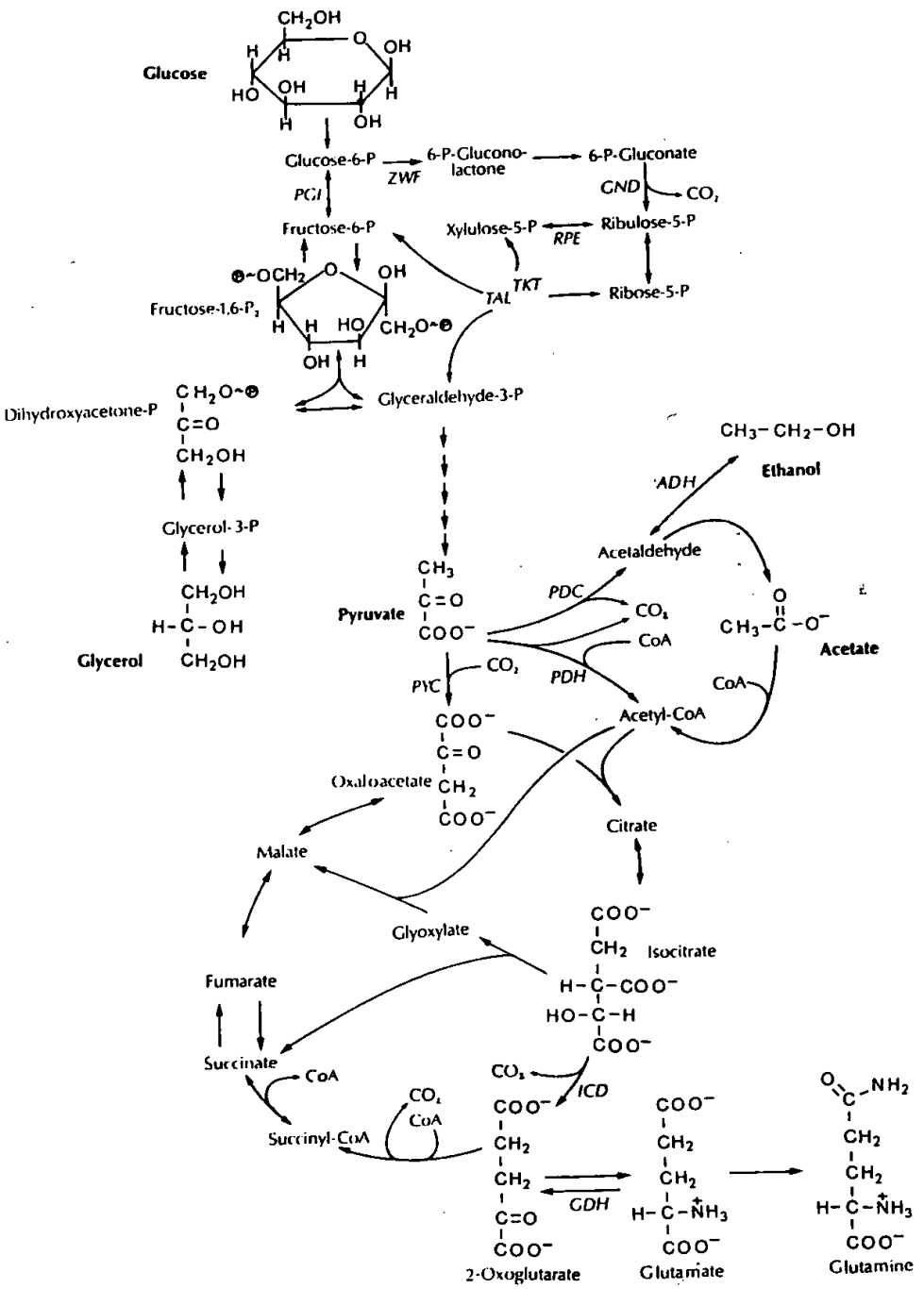

Fig. 1. The pathways of catabolism of glucose in *S. cerevisiae*. The figure is redrawn from a version by Fraenkel (1982). The structures of all of the intermediates and products discussed in the text are given. Only the β anomer of the substrate D-glucose is drawn. The ^{13}C label at C-2 of glucose is marked by (●). Enzymes which are specifically mentioned are identified by three-letter acronyms as follows: *ZWF*, glucose-6-phosphate dehydrogenase; *PGI*, phosphoglucose isomerase; *GND*, 6-phosphogluconate dehydrogenase; *RPE*, ribulose-5-phosphate 3-epimerase; *TAL*, transaldolase; *PDC*, pyruvate decarboxylase; *ADH*, alcohol dehydrogenase; *PDH*, pyruvate dehydrogenase; *ICD*, isocitrate dehydrogenase; *GDH*, glutamate dehydrogenase. Irreversible reactions are denoted by single-headed arrows. The presence of two arrows indicates a separate enzyme for each direction of a reaction. Reversible reactions are represented by bi-directional arrows (except, for simplicity, the four reversible reactions 'below' glyceraldehyde-3-phosphate).

gene (which encodes glucose-6-phosphate dehydrogenase) blocks suppression of a *pgi1Δ* mutation. This is also highly indicative of the importance of the hexose monophosphate pathway, but it must be stressed that, to date, none of the studies have proved that the suppression of a *pgi1* mutation actually involves a metabolic bypass in which glucose is catabolized by means of increased flux via the hexose monophosphate pathway; i.e. the flow of carbon from substrate to product(s) has not been demonstrated. One could postulate three possible metabolic routes. Firstly, increased flux via the hexose monophosphate pathway. Secondly, use of the glycolytic sequence involving an isoenzyme of phosphoglucose isomerase encoded by a gene which is normally cryptic. This is not without precedent in *S. cerevisiae*: for example *ADH4* (encoding an isoenzyme of ethanol dehydrogenase) was discovered in *adh1 adh2 adh3* triple mutants (Paquin & Williamson, 1986). Thirdly, the use of a hitherto-unknown pathway of glucose catabolism. This concept has also been suggested previously to explain the bypass of *pfk1 pfk2* double mutants which lack phosphofructokinase (Breitenbach-Schmitt *et al.*, 1984). ^{13}C nuclear magnetic resonance (NMR) spectroscopy is a very good technique for metabolic studies (London, 1988; Davies & Brindle, 1992); hence it was decided to use ^{13}C-labelled glucose to analyse the patterns of metabolism by ^{13}C NMR in suppressed *pgi1Δ* mutants. Providing yeast with [2-^{13}C]glucose allows one to discriminate between the known pathways as described below.

Use of the hexose monophosphate pathway will give rise to fructose 1,6-bisphosphate with label at C-1 and C-3. Subsequent cleavage by aldolase would yield dihydroxyacetone phosphate labelled at C-1 and C-3, which would be metabolized by the conventional glycolytic sequence to produce pyruvate labelled at C-3 and C-1, respectively. This labelled pyruvate has three possible fates, the first being conversion to ethanol (labelled at C-2) and $^{13}CO_2$ via pyruvate decarboxylase and ethanol dehydrogenase (Fig. 1). Since it is common for ethanolic fermentation to be accompanied by the formation of some glycerol (to preserve the NAD/NADH balance) one would also expect to see glycerol labelled at C-1 and C-3 which was derived from the C-1,3-labelled dihydroxyacetone phosphate (Fig. 1). A second possible fate of the pyruvate would be via pyruvate dehydrogenase, which would result in loss of $^{13}CO_2$ and formation of [2-^{13}C]acetate. Subsequent metabolism of the [2-^{13}C]acetate via the tricarboxylic acid and/or glyoxylate cycles would give predictable labelling patterns as described previously (Dickinson *et al.*, 1983; Dickinson & Hewlins, 1991) when the fate of [2-^{13}C]acetate was analysed by ^{13}C NMR. The third possible route for metabolism of some pyruvate would be via pyruvate carboxylase to 1,3-labelled oxaloacetate. The ^{13}C at C-1 of oxaloacetate would be lost as $^{13}CO_2$ at the isocitrate dehydrogenase step of the tricarboxylic acid cycle and the remaining label would result in glutamate labelled only at C-2 – an isotopomer that would be easy to identify, but has never been seen in yeast. Metabolism of isocitrate via the glyoxylate cycle would give readily identifiable intermediates.

The expression of a previously cryptic gene resulting in a new phosphoglucose isomerase activity would give catabolism of [2-^{13}C]glucose via a completely reinstated glycolytic sequence. This would be expected to produce [2-^{13}C]pyruvate and hence ethanol labelled at C-1 along with some [2-^{13}C]glycerol (Fig. 1). Oxidative decarboxylation of this [2-^{13}C]pyruvate via pyruvate dehydrogenase would give [1-^{13}C]acetate, which could enter the tricarboxylic acid cycle to yield glutamate labelled at C-1 on the first turn of the cycle and thence at C-5 on the second turn. The labelling of glutamate at C-1 and C-5 would yield a very characteristic spectrum. Metabolism of the [1-^{13}C]acetate to [6-^{13}C]isocitrate would give rise via the glyoxylate cycle to glutamate labelled only at C-1 on each successive turn of that cycle. The conversion of [2-^{13}C]pyruvate to [2-^{13}C]oxaloacetate by pyruvate carboxylase would give rise to glutamate labelled at C-3 on the first turn of the tricarboxylic acid cycle, 2,3- on the second turn and 1,2,3- on the third and subsequent turns of that cycle. This would also be a unique labelling pattern, which, even if it was combined with the formation of 1-, and 1,5-labelled glutamate, would still be very different from patterns which have been recorded previously. Finally, conversion of any [2-^{13}C]oxaloacetate to [3-^{13}C]isocitrate and subsequent intervention of the glyoxylate cycle would yield glutamate labelled only at C-2 and C-3. Hence, the catabolism of [2-^{13}C]glucose via all known metabolic routes (Fig. 1) produces predictable labelling patterns in intermediates whose constituent carbon atoms have well-defined resonances in the NMR spectrum. With this knowledge we embarked upon an NMR analysis of the metabolism of glucose in a *pgi1Δ* mutant carrying a *spg29* suppressor mutation which allows growth on glucose.

METHODS

Media, strains and genetic methods. Cultural conditions and complex growth media have been described previously (Dickinson, 1991). Complex media contained, per litre: yeast extract (10 g), bacteriological peptone (20 g), adenine (0·1 g), uracil (0·1 g) and the specified carbon source (YEPD, 20 g D-glucose; YEPF, 20 g D-fructose). Minimal medium with fructose (2%, w/v) as major carbon source was supplemented with 0·1% (w/v) glucose. Standard genetic techniques were used for mating, sporulation and dissection (Sherman, 1975; Mortimer & Hawthorne, 1975). *S. cerevisiae* strain AAG2 (*MATα ura3 his4 can pgi1Δ25*::*LEU2*) was obtained from A. Aguilera (University of Seville, Spain). Strain S9 (*MATα ura3 his4 can pgi1Δ25*::*LEU2 spg29-1*) was derived by crossing E9 (obtained as a spontaneous revertant of AAG2 as described below) to strain 329 (*MAT*a *ade8*) and repeated backcrossing of appropriate *MAT*a segregants to AAG2. Strain OH1 (*MAT*a *ura3 his4 can*) is a wild-type (i.e. *PGI1*) haploid derived from a cross between 329 and AAG2. Strains 26.1A (*MAT*a *ade1 ade2 his7 pgi1-1*) and 26.6B (*MATα ura1 pgi1-1*) were constructed from mutant 9520b/TIC (*MATα pgi1-1*) (Maitra, 1971), which was provided by P. K. Maitra (Tata Institute of Fundamental Research, Bombay, India).

Isolation of mutants carrying suppressors of *pgi1Δ* mutations. A 100 ml batch of strain AAG2 was grown overnight in YEPF to OD_{600} 1·00. The cells were then harvested by

centrifugation and resuspended in 100 ml YEPD. The culture was divided into 10 separate aliquots, each of which was incubated in a 100 ml conical flask at 30 °C for 6 d. After this time 0·1 ml samples were removed from each small culture and plated directly onto YEPD. The YEPD plates were inspected after 3, 5 and 7 d. Colonies which had appeared at each time and were large enough to handle were transferred to YEPF, incubated for 4–5 d and then replica-plated to YEPF and YEPD. Clones which were able to grow well on YEPD when re-tested were retained for further analysis.

NMR analyses. For *in vivo* NMR analysis cells of S9 were grown in YEPD to OD_{600} 4·0 (late exponential phase for this strain), harvested by centrifugation, resuspended in 2 ml 50 mM potassium phosphate buffer pH 6·0 containing 30 % (v/v) ^2H_2O and transferred to a 10 mm NMR tube with a gassing system which provides both aeration and mixing (described by Lloyd *et al.*, 1993). Then 70 mg [2-^{13}C]glucose (99 atom % enrichment) was added and ^{13}C data were collected in blocks of 500 accumulations (each of 8K data points over 22000 Hz) with an acquisition time of 0·18 s and a 1 s delay between pulses using a Bruker WM360 spectrometer operating at 90·5 MHz. High-power ^{1}H-decoupling was used during acquisition, with low-power decoupling during delays: preliminary experiments had determined the correct settings to effect proton decoupling with sufficient dielectric heating to maintain a temperature of 30 °C ± 1 °C.

Metabolic analyses were also done on cells which had been grown to OD_{600} 4·0 in YEPD, harvested by centrifugation and resuspended in YEP containing [2-^{13}C]glucose. After 150 min incubation at 30 °C cells were harvested and perchloric acid extracts prepared for ^{13}C NMR analysis as described previously (Dickinson & Hewlins, 1988, 1991). The spectrum was recorded for the solution in a 5 mm NMR tube, using 32K data points over 22000 Hz, with broad-band ^{1}H-decoupling, and also by the DEPT method to determine the number of protons attached to each carbon signal. A two-dimensional ^{13}C–^{1}H chemical shift correlation spectrum was carried out using the XHCORRD routine in the standard Bruker NMR software. All chemical shifts are reported in p.p.m. relative to the signals (^{1}H and ^{13}C as appropriate) from sodium 3-(trimethylsilyl)propane-1-sulphonate (assigned $\delta = 0$ for both nuclei) measured in ^2H_2O solution as the external standard.

Enzyme assays. Cells were harvested by centrifugation, resuspended in 50 mM potassium phosphate buffer pH 7·4 containing 2 mM EDTA and 2 mM 2-mercaptoethanol and disrupted using a Braun homogenizer as described by Dickinson & Williams (1986). Aliquots of this homogenate were used immediately as the source of enzyme. Phosphoglucose isomerase (EC 5.3.1.9), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and gluconate-6-phosphate dehydrogenase (EC 1.1.1.43) were assayed as described by Maitra & Lobo (1971). Ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) was assayed according to Williamson & Wood (1966). Transaldolase (EC 2.2.1.2) was assayed by the method of Tchola & Horecker (1966). NAD- and NADP-dependent glutamate dehydrogenases (EC 1.4.1.2 and 1.4.1.4) were both assayed in 0·1 M imidazole buffer pH 7·9 in a total volume of 1·8 ml. NADP-dependent glutamate dehydrogenase was assayed by following NADPH disappearance when 2-oxoglutarate (22·8 µmol) was converted to glutamate in the presence of ammonium acetate (614 µmol) using 0·03 µmol NADPH. NAD-dependent glutamate dehydrogenase was monitored by following the increase in absorbance at 340 nm due to NADH formation when glutamate (25 µmol) was converted to 2-oxoglutarate using 0·375 µmol NAD.

RESULTS

Isolation and genetic characterization of mutants carrying suppressors of *pgi1Δ* mutations

The spontaneous mutants which arose on YEPD after 3 d were called 'early mutants' and numbered E1–E33. The 'mid mutants' collected after 5 d were numbered M1–M14 and the 'late mutants' were numbered L1–L29. There was no correlation with the original time of appearance of an individual mutant and its rate of proliferation when examined on YEPD plates or in YEPD liquid medium: i.e. there was a range of generation times amongst all three sets of mutants. However, all of the 'mid mutants' subsequently died over the next 4 weeks (irrespective of whether they were stored on YEPD or YEPF) before they had been characterized genetically. Nevertheless, the frequency of suppressor mutations would seem to be 10^{-7}–10^{-8}. Sixteen of the remaining mutants which grew most rapidly on YEPD were mated on YEPF to strain 26.1A. The diploids which formed were selected on fructose minimal medium supplemented with 0·1 % glucose. These diploids were then replicated onto YEPD: none of them grew on YEPD, showing that the suppressor mutations were all recessive. A series of outcrosses to suitable wild-type haploids allowed a subsequent complementation analysis, which indicated the existence of at least three distinct *spg* mutations. It seemed prudent to retain the terminology of Aguilera (1987) for such mutants rather than add a further acronym to an already crowded literature of phenotypes. However, to avoid the possibility of a future overlap of mutants from our different laboratories it was decided to call these three mutations *spg29* to *spg31*. It was not possible to

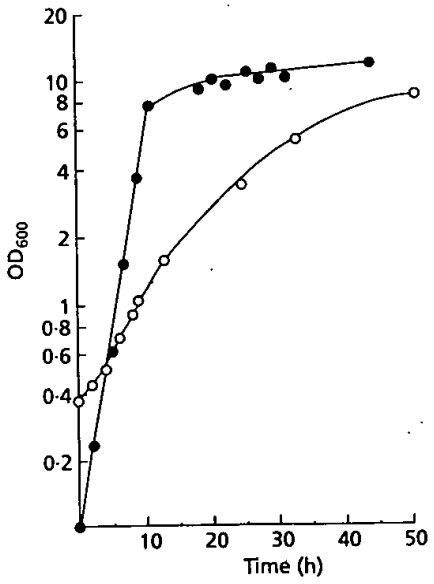

Fig. 2. Typical growth curves for S9 (*pgi1Δ spg29-1*; ○) and wild-type haploid OH1 (*PGI1*; ●) in YEPD liquid medium. Both strains were inoculated into fresh pre-warmed medium from starter cultures grown to exponential phase in YEPD.

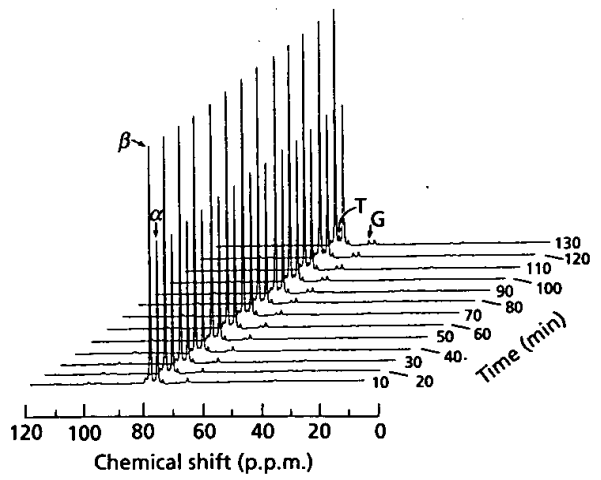

Fig. 3. Time course of utilization of [2-^{13}C]glucose by strain S9. *In vivo* time-elapsed proton decoupled NMR spectra are shown. α, C-2 of α-D-glucose; β, C-2 of β-D-glucose; T, C-2 of trehalose; G, C-1,3 glycerol.

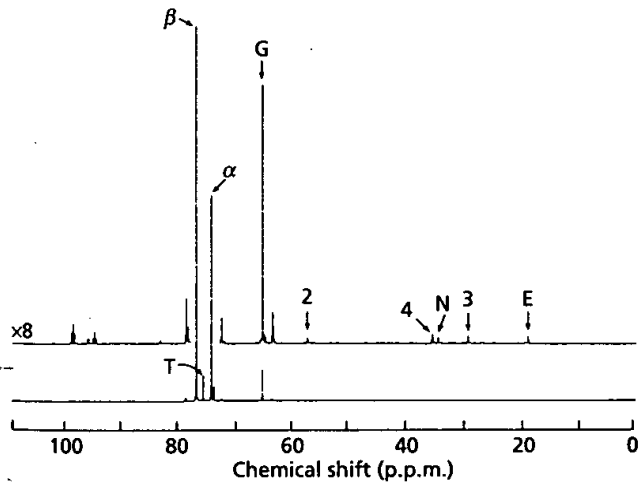

Fig. 4. ^{13}C NMR spectrum of a perchloric acid extract of strain S9 made after growth in YEP-[2-^{13}C]glucose. E, C-2 of ethanol; 4, C-4 of glutamate; 3, C-3 of glutamate; 2, C-2 of glutamate; N, C-4 of glutamine; other resonances marked are as in Fig. 3.

definitely assign all of the mutants, mainly due to their different rates of proliferation on YEPD prior to mating. The *spg29* mutation was represented by three alleles all resulting in very similar phenotypes, the most prominent of which was the most rapid growth (generation time of 335 min in exponential phase) in glucose (Fig. 2).

^{13}C NMR analysis of metabolism in a *pgi1Δ spg29* mutant

In vivo NMR analysis of strain S9 showed that the major metabolic product derived from [2-^{13}C]glucose was glycerol labelled at C-1 and C-3 (Fig. 3). This signal corresponding to glycerol (65·3 p.p.m.) increased with time as did the resonance corresponding to C-2 of the storage compound trehalose (75·7 p.p.m.). Three other resonances can be seen in Fig. 3 (78·6 p.p.m., 72·4 p.p.m. and 63·5 p.p.m.); they are explained below. The *in vivo* experiment was not run for longer because although it could be seen (from comparison of the peak heights of the signals due to C-2 of glucose) that very little glucose had been metabolized, the experiment had served its purpose, i.e. identification of the metabolic pathway by which the glucose was catabolized. Detailed isotopomer analysis is always better done on perchloric acid extracts because field inhomogeneity within the sample of *in vivo* experiments leads to signal broadness.

One possible criticism of the *in vivo* NMR study above is that the cells are in a non-growing situation because no nitrogen source was provided, although other workers actually strive to devise conditions of rigorous non-growth for metabolic analysis (e.g. Benevolensky *et al.*, 1994). However, in this case the criticism would seem to be irrelevant because analysis of an extract made from cells grown in YEP-[2-^{13}C]glucose gave a similar result (Fig. 4). In addition, the expanded spectrum allows the identification of resonances at 18·9 p.p.m. (ethanol C-2), 29·4 p.p.m. (glutamate C-3), 35·6 p.p.m. (glutamate C-4) and 57·3 p.p.m. (glutamate C-2). Although small, the signal due to ethanol C-2 is highly significant because it confirms that ^{13}C in this molecule has been derived via the hexose monophosphate pathway (as explained in the Introduction). The fact that the intensity of the signal for glutamate C-4 is significantly greater than that for C-3, which in turn is much greater than the intensity of the glutamate C-2 resonance, is also important because these data indicate that glutamate is being labelled as predicted for metabolism of [2-^{13}C]glucose around the hexose monophosphate pathway to yield pyruvate labelled at C-3 and C-1. This is decarboxylated to [2-^{13}C]acetate, which then enters the tricarboxylic acid cycle. Indeed, it is just possible to observe the appearance of label in the C-4 position of glutamate first, followed by label at C-3 and C-2, in the time-elapsed spectra in Fig. 3. The resonance at 34·6 p.p.m. is due to C-4 of glutamine formed directly from glutamate. The multiplets at 98·6 p.p.m. and 94·8 p.p.m. are respectively C-1 of β-D-glucose and α-D-glucose which are visible mainly due to natural abundance of ^{13}C at this position. The singlet (centre line)resonance in each case corresponds to glucose labelled only at C-1; the doublets are due to interaction with the heavily labelled C-2. Similarly, the resonance at 63·5 p.p.m. seen here and noted in Fig. 3 along with the resonances at 78·6 p.p.m. and 72·4 p.p.m. are due to glucose C-6, C-3 and C-4 respectively: all were observed due to natural abundance.

Initial signal assignment was made by comparison of ^{13}C chemical shifts with those of standard compounds recorded under comparable conditions. Additional information was obtained from further NMR experiments carried out on the perchloric acid extract. The DEPT method was used to establish the number of protons attached to each ^{13}C nucleus and this confirmed CH or CH_2 for each signal in accordance with the assignments

Table 1. **Specific activities of various enzymes in strains AAG2 (*pgi1Δ*) and S9 (*pgi1Δ spg29*)**

Strain S9 was grown to stationary phase in YEPD. Strain AAG2 was grown to stationary phase in YEPF, harvested and transferred to YEPD for 24 h. The results are the means of duplicate determinations.

Enzyme*	Specific activity [mU (mg protein)$^{-1}$]		Activity in suppressed strain relative to parent (%)
	AAG2	S9	
G6PDH	206·5	194·3	94·1
GND	81·9	169·1	206·5
RPE	1·0	2·1	200·7
TAL	74·4	117·6	158·0
NAD-GDH	24·4	57·2	234·5
NADP-GDH	42·7	86·3	202·0

*G6PDH, glucose-6-phosphate dehydrogenase; GND, 6-phosphogluconate dehydrogenase; RPE, ribulose-5-phosphate 3-epimerase; TAL, transaldolase; NAD-GDH, NAD-dependent glutamate dehydrogenase; NADP-GDH, NADP-dependent glutamate dehydrogenase.

given above. Further information was obtained from the ^{13}C–^{1}H correlation spectrum. In particular this confirmed that the ^{13}C at 65·3 is attached to protons at δH 3·47 and 3·55, as expected for glycerol. The carbon signals attributed to ethanol C-2, glutamate C-4, glutamine C-4 and β-D-glucose C-1 were shown to be connected to protons having chemical shifts of 1·3, 2·33, 2·45 and 4·6 respectively in accord with standard values.

Enzyme assays on the *pgi1Δ* parent and the *pgi1Δ spg29* suppressed double mutant

Phosphoglucose isomerase was undetectable in the parental strain AAG2 and in strain S9, confirming that both strains carry the *pgi1Δ* mutation and that suppression in S9 was not due to the regaining of this activity. The specific activity of glucose-6-phosphate dehydrogenase was virtually identical in both strains, but the specific activities of all of the other enzymes assayed were all increased in the bypass mutant to 150–235% of the levels present in the parental strain (Table 1). The greatest increase in specific activity occurred in the case of NAD-dependent glutamate dehydrogenase.

DISCUSSION

Spontaneous mutants were isolated carrying mutations which suppress the *pgi1Δ* mutation, thus allowing growth on glucose. In many respects these newly isolated *spg* mutants seem similar to those described by Aguilera (1987) and also the *rgl* mutants of Gamo *et al.* (1993). The reason for isolating these mutants was to establish the metabolic pathway used which enables a *pgi1Δ* suppressed mutant to utilize glucose. ^{13}C NMR studies clearly showed that the hexose monophosphate pathway was used to suppress the defect in phosphoglucose isomerase. Despite the fact that previous studies all suggested the hexose monophosphate pathway was used in suppressed *pgi1* strains (Aguilera, 1987; Gamo *et al.*, 1993; Boles *et al.*, 1993), this is the first time that the flux of carbon has actually been proved.

The specific activities of enzymes of the hexose monophosphate pathway were increased in the suppressed strain compared with the parental *pgi1Δ* strain with the exception of glucose-6-phosphate dehydrogenase. These results serve to confirm the NMR study and lead to the conclusion that glucose-6-phosphate dehydrogenase is not the limiting step of the metabolic bypass. Aguilera (1987) reported no significant differences in specific activities between *pgi1Δ* and *pgi1Δ spg1* double mutants. He also reported the specific activity of 6-phosphogluconate dehydrogenase as lower in the suppressed strains than in the *pgi1Δ* parents whereas we observed that the specific activity of this enzyme was double compared with the parental strain. The reason for the difference between our results is likely to be because Aguilera grew both sorts of strain in YEP containing 2% fructose and 0·1% glucose, whilst we grew the *pgi1Δ* strain in this medium (it will not grow in YEPD), and then transferred it to YEPD for 24 h before determining enzyme activities; mutant S9 was grown in YEPD. The very reason for doing this was the possibility that the activity of one or more enzymes of the hexose monophosphate pathway might be higher in the presence of (2%) glucose than (2%) fructose.

The largest increase recorded for a single enzyme was for NAD-dependent glutamate dehydrogenase. This observation confirms the work of Boles *et al.* (1993) on the importance of this activity in suppressing *pgi1Δ* mutations. However, it is worth commenting that the elevation of NAD-dependent glutamate dehydrogenase is nothing like as high as the massive overexpression achieved by Boles *et al.* (1993), who put the *GDH2* gene in a multi-copy plasmid. As demonstrated in the present study, *S. cerevisiae* does not have to achieve such a dramatic alteration to its normal metabolic activities: production of the large amounts of glycerol which were observed serves as an additional route for NAD regeneration. It is conceivable that other *spg* mutations result merely in the production of more glycerol without any increase whatsoever in the activity of NAD-dependent glutamate dehydrogenase; although it is not clear whether the extra glutamate produced (and not recycled to 2-oxoglutarate by NAD-dependent glutamate dehydrogenase) would be an advantage that allowed more rapid growth (glutamate is the major pool of nitrogen), or a detriment to the cell because of the depletion of an important intermediate of the tricarboxylic acid cycle.

In a study of carbon metabolism during sporulation in acetate it was noted that at a distinct time in the process metabolic flux around the hexose monophosphate pathway increased appreciably (Dickinson *et al.*, 1983). Per-

haps this up-regulation in sporulating diploids involves a gene which is mutated in *spg* mutants. Cloning of *SPG* genes will allow an examination whether the same genes are involved in controlling expression of hexose monophosphate pathway functions in sporulation and glycolytic bypass mutants.

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2) Microbiology 1995 Feb;141 (Pt 2):385-91
In Saccharomyces cerevisiae deletion of phosphoglucose isomerase can be suppressed by increased activities of enzymes of the hexose monophosphate pathway.
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3) J Bacteriol 1997 Apr;179(8):2724-30
Interruption of the phosphoglucose isomerase gene results in glucose auxotrophy in Mycobacterium smegmatis.
Tuckman D, Donnelly RJ, Zhao FX, Jacobs WR, Connell ND

4) Eur J Biochem 1993 Oct 1;217(1):469-77
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Boles E, Lehnert W, Zimmermann FK

5) Appl Environ Microbiol 1991 Oct;57(10):2995-9
Deletion of pgi alters tryptophan biosynthesis in a genetically engineered strain of Escherichia coli.
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Thank you,
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Eur. J. Biochem. 217, 469–477 (1993)

The role of the NAD-dependent glutamate dehydrogenase in rest ring growth on glucose of a *Saccharomyces cerevisiae* phosphoglucose isomerase mutant

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Phosphoglucose isomerase *pgi1*-deletion mutants of *Saccharomyces cerevisiae* cannot grow on glucose as the sole carbon source and are even inhibited by glucose. These growth defects could be suppressed by an over-expression on a multi-copy plasmid of the structural gene *GDH2* coding for the NAD-dependent glutamate dehydrogenase. *GDH2* codes for a protein with 1092 amino acids which is located on chromosome XII and shows high sequence similarity to the *Neurospora crassa* NAD–glutamate dehydrogenase. Suppression of the *pgi1* deletion by over-expression of *GDH2* was abolished in strains with a deletion of the glucose-6-phosphate dehydrogenase gene *ZWF1* or gene *GDH1* coding for the NADPH-dependent glutamate dehydrogenase. Moreover, this suppression required functional mitochondria. It is proposed that the growth defect of *pgi1* deletion mutants on glucose is due to a rapid depletion of NADP which is needed as a cofactor in the oxidative reactions of the pentose phosphate pathway. Over-expression of the NAD-dependent glutamate dehydrogenase leads to a very efficient conversion of glutamate with NADH generation to 2-oxoglutarate which can be converted back to glutamate by the NADPH-dependent glutamate dehydrogenase with the consumption of NADPH. Consequently, over-expression of the NAD-dependent glutamate dehydrogenase causes a substrate cycling between 2-oxoglutarate and glutamate which restores NADP from NADPH through the coupled conversion of NAD to NADH which can be oxidized in the mitochondria. Furthermore, the requirement for an increase in NADPH consumption for the suppression of the phosphoglucose isomerase defect could be met by addition of oxidizing agents which are known to reduce the level of NADPH.

Glycolysis plays a fundamental role in the degradation of fermentable carbon sources in the yeast *Saccharomyces cerevisiae* providing the cell with metabolic energy and intermediates. Phosphoglucose isomerase is the second enzyme in the glycolytic pathway, interconverting glucose 6-phosphate and fructose 6-phosphate. Maitra (1971) was the first to obtain mutants of the structural gene *PGI1* and found that they could not grow on glucose. Aguilera (1986) confirmed this for a deletion mutant with no residual activity. This was an unexpected result because the direct oxidation of glucose 6-phosphate via the pentose phosphate pathway should allow to supply yeast cells with sufficient energy and intermediate metabolites to support at least slow growth. Phosphoglucose-isomerase-lacking mutants of *Kluyveromyces lactis* (Goffrini et al., 1991) and also of *Escherichia coli* (Vinopal et al., 1975) can still grow on glucose.

On the other hand, phosphoglucose isomerase deletion mutants of *S. cerevisiae* cannot grow on a pure fructose medium because the phosphoglucose isomerase reaction is the only step catalysing the interconversion of fructose 6-phosphate to glucose 6-phosphate which is an essential metabolite (Aguilera, 1986; Boles et al., 1993). *pgi1* mutants can grow on media containing fructose and not more than 0.2% glucose, higher glucose concentrations inhibit growth (Maitra, 1971; Ciriacy and Breitenbach, 1979; Aguilera, 1986). Maitra (1971) assumed that growth inhibition results from an accumulation of toxic concentrations of glucose 6-phosphate. Ciriacy and Breitenbach (1979) suggested that ATP depletion is the cause for the glucose sensitivity.

Aguilera (1987) selected *pgi1* suppressor mutants restoring growth on synthetic media with 2% glucose as the sole carbon source. Recently, Gamo et al. (1993) isolated *pgi1* suppressor mutants insensitive to glucose inhibition by selection on media containing 2% fructose and 2% glucose. Suppression of the *pgi1* defect depended on a functional respiratory system. In both cases, the suppressor mutations were interpreted to allow *S. cerevisiae pgi1* mutant strains to enhance glucose catabolism through the pentose phosphate pathway and a complete respiratory breakdown. However, the actual functions of the suppressor genes were not identified.

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Abbreviations. Glc6PDH, glucose-6-phosphate dehydrogenase; NAD-GluDH and NADPH-GluDH, NAD- and NADPH-dependent glutamate dehydrogenase; PCR, polymerase chain reaction; ORF, open reading frame.

Enzymes. Phosphoglucose isomerase (EC 5.3.1.9); glucose-6-phosphate dehydrogenase (EC 1.1.1.49); NADPH-dependent glutamate dehydrogenase (EC 1.4.1.4); NAD-dependent glutamate dehydrogenase (EC 1.4.1.2).

Note. The nucleotide sequence of the *GDH2* gene published here has been deposited with the EMBL/GenBank sequence data bank and is available under the accession number X72015.

We prepared a yeast genomic library from a *pgi1* deletion mutant (Boles and Zimmermann, 1993a) in the multi-copy vector YEplac181 (Gietz and Sugino, 1988), transformed it back into the deletion mutant and tested the transformants for growth on a mineral salts/glucose medium. One of the glucose-positive transformants carried the structural gene *GDH2* coding for the NAD-dependent glutamate dehydrogenase. Further investigations showed that this over-expression creates a cyclic transhydrogenase system between the NADPH- and the NAD-dependent glutamate dehydrogenases, converting NADPH to NADH and replenishing the pool of NADP which is required for the oxidative reactions of the pentose phosphate pathway.

MATERIALS AND METHODS

Yeast strains and growth conditions

The isogenic wild-type strains ENY. WA-1A (*MATa, ura3-52, leu2-3,112, trp1-289, his3-delta1, MAL2-8^c, MAL3, SUC3*) and ENY. WA-1B (*MATa, ura3-52, leu2-3,112, trp1-289, HIS3, MAL2-8^c, MAL3, SUC3*) were kindly provided by K. D. Entian (University of Frankfurt). All mutants used in this work were derived from these strains unless otherwise stated. Strains EBY23 (*pgi1Δ::URA3*) and EBY8 (*fba1Δ::URA3*) were described by Boles and Zimmermann (1993a), EBY812 (*pfk1Δ::LEU2Δ, pfk2::URA3*) by Boles et al. (1993) and EBY88 (*tpi1Δ::HIS3*), EBY71 (*pgk1Δ::LEU2*), EBY66 (*gpm1Δ::LEU2*), EBY55 (*pyk1Δ::LEU2*) by Boles and Zimmermann (1993b). Strain EBY. UTL-23 (*pgi1Δ::URA3*) was derived from strain UTL-7A (*MATa, leu2-3,112, ura3-52, trp1*) (Corominas et al., 1992). Yeast cells were grown at 28°C in yeast/peptone media (1% yeast extract, 2% bactopeptone), in synthetic minimal media [0.17% Difco yeast nitrogen base, 0.5% $(NH_4)_2SO_4$, 10 mM KH_2PO_4, pH 6.2, supplemented for auxotrophic demands] or synthetic complete media with different carbon sources (Boles and Zimmermann, 1993a, b).

Molecular biology techniques

DNA was prepared and manipulated according to the procedures described in Sambrook et al. (1989). Yeast-specific techniques were described by Guthrie and Fink (1991). pUC18, pUC19 (Yanisch-Perron et al., 1985), pBluescript II SK+ (Stratagene GmbH) and the plasmids YEplac181, YEplac112 and YEplac195 from the series of Gietz and Sugino (1988) served as vectors. *Escherichia coli* strains JM101 and SURE (Stratagene GmbH) were used for the propagation of plasmids. Plasmids were transformed into yeast according to Schiestl and Gietz (1989) and re-isolated from yeast as previously described by Boles and Zimmermann (1993a).

Construction of a genomic gene library

Yeast chromosomal DNA was prepared from strain EBY23 (*pgi1Δ*) according to Ciriacy and Williamson (1981). Chromosomal DNA (100 µg) was partially digested with 3 units of restriction enzyme *Sau*3A for 1 min at 37°C. DNA restriction fragments were electrophoresed in an agarose gel and fragments with a size between 4.5 and 10 kb were isolated from the gel. Isolated DNA fragments (9 µg) were ligated with 1 µg *Bam*HI-digested vector YEplac181 which had been dephosphorylated using calf intestinal phosphatase (Boehringer, Mannheim). The ligation reaction was transformed into *E. coli* strain SURE cells which were plated onto selective media with ampicillin, isopropyl -thiogalactoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside to allow blue-white screening. There were 5000 white among a total of 11000 colonies which were washed from the plates and plasmids prepared using three Qiagen tip100 resins (Diagen GmbH).

Chromosome mapping

Gel blots with chromosome-size DNAs of *S. cerevisiae* strain FY23/6 separated in a pulsed-field gel were obtained from Boehringer (Mannheim).

DNA sequencing

Double-stranded templates were sequenced by the dideoxynucleotide chain-terminating method (Sanger et al., 1977) using the Pharmacia T7 sequencing kit. To obtain overlapping sequencing priming sites throughout the whole sequence of the insert of YEpMSP3, the transposon-based DNA sequencing strategy described by Strathmann et al. (1991) was employed using the TN1000 kit obtained from Angewandte Gentechnologie Systeme GmbH. The sequence of the complete 4.8-kb insert of YEpMSP3 was determined for both strands. The DNASIS/PROSIS program (Pharmacia) was used for DNA and protein analysis. Sequence comparisons were made by using CD-ROM and the HIBIO™ gene/protein sequence database (Hitachi).

Cloning of the *URE2* gene

The *URE2* gene was cloned by the polymerase chain reaction (PCR; Saiki et al., 1985) using a pair of primers (Roth) designed to PCR-amplify a DNA fragment enclosing part of the coding region of the *URE2* gene. One oligonucleotide (5′-AACAATAACAGCGGCCG-3′) is located at position 359–375 of the published sequence (Coschigano and Magasanik, 1991) just upstream of a *Not*I restriction site. The second oligonucleotide (5′-ATGCGAATTCTATCCACGAC-ATT-3′) is located at position 1155–1133. It contains a single G→C base exchange at the fifth position as compared to the published sequence to create an *Eco*RI restriction site. PCR with *Taq* polymerase (Boehringer, Mannheim) with this oligonucleotide pair as primers and chromosomal DNA of strain ENY. WA-1A as template yielded a 0.8-kb fragment containing part of the coding region of *URE2*. The fragment was then digested with *Eco*RI and *Not*I and cloned into the pBluescript II SK+ vector, resulting in plasmid pURE2.

Construction of plasmids

YEpMSP3-T: a 4.8-kb *Sst*I–*Xba*I DNA fragment of plasmid YEpMSP3 containing the complete insert was recloned into vector YEplac112.

YEpmsp3-B: a 2.9-kb *Bam*HI–*Sph*I DNA fragment containing only the first 2948 bp of the insert of plasmid YEpMSP3 was subcloned into YEplac181.

YEpmsp3-X: a 3.9-kb *Xho*I–*Sst*I fragment containing the last 3931 bp of the insert of plasmid YEpMSP3 was subcloned into YEplac181.

YEpGDH1-U: a 2.4-kb *Pst*I–*Cla*I fragment of plasmid pYC1 (Moye et al., 1985) containing the complete *GDH1* gene was subcloned into YEplac195.

pURE2del (*ure2Δ::LEU2*): the 0.3-kb *Sph*I−*Sca*I fragment of plasmid pURE2 containing part of the *URE2* coding region from +392 bp to +670 bp according to the published sequence (Coschigano and Magasanik, 1991) was replaced by a 2.5-kb *Sph*I−*Sma*I fragment with the *LEU2* gene originally cloned into pUC18.

pGDH1del (*gdh1Δ::URA3*): a 5.4-kb *Bam*HI−*Cla*I fragment of plasmid pYC1 (Moye et al., 1985) containing the *GDH1* gene was subcloned into pBluescript II SK+, resulting in pGDH1. A 1.0-kb *Hin*dIII−*Stu*I fragment of pGDH1 containing most parts of the *GDH1* coding region from +23 bp to +976 bp according to the published sequences (Moye et al., 1985; Nagasu and Hall, 1985) was replaced by a 1.1-kb *Hin*dIII−*Sma*I fragment with the *URA3* gene of plasmid YEp24 (Botstein et al., 1979).

Construction of deletion strains

The deletion mutants were constructed following the one-step gene replacement procedure of Rothstein (1983). The deletion plasmids carried different selectable markers which allowed us to construct multiple deletions in the same recipient yeast strain. pEB22 (*pgi1Δ::TRP1*) (Boles et al., 1993) was digested with *Bam*HI and *Sac*I and transformed into the haploid strain ENY. WA-1B selecting for tryptophan prototrophy on a 2% fructose and 0.1% glucose medium, resulting in strain EBY22 (*pgi1Δ::TRP1*). The deletion plasmid pM19-7 (*zwf1Δ::URA3*) of Thomas et al. (1991), which contains most parts of the open reading frame of the *ZWF1* gene replaced by the *URA3* gene, was cut by *Hin*dIII and *Bam*HI and used to transform strain EBY22 to uracil prototrophy on a 2% fructose and 0.05% glucose medium, resulting in strain EBY228 (*pgi1Δ::TRP1, zwf1Δ::URA3*). pURE2del (*ure2Δ::LEU2*) was digested with *Not*I and *Eco*RI (partially) and transformed into strain EBY22 selecting for leucine prototrophy on a 2% fructose and 0.05% glucose medium, resulting in strain EBY229 (*pgi1Δ::TRP1, ure2Δ::LEU2*). pGDH1del (*gdh1Δ::URA3*) was digested with *Pvu*II and *Cla*I and used to transform the haploid wild-type strain ENY. WA-1A to uracil prototrophy, resulting in strain EBY99 (*gdh1Δ::URA3*). Strain EBY99 was then crossed with strain EBY22 and tetrad analysis was performed, resulting in strain EBY227 (*pgi1Δ::TRP1, gdh1Δ::URA3*) which did grow on a 2% fructose and 0.05% glucose medium. Deletion mutants were confirmed by Southern blot analysis, enzyme activity assay and their growth properties.

Enzyme assays

Crude extracts were prepared using glass beads for breaking the cells as described by Ciriacy and Breitenbach (1979). Phosphoglucose isomerase activity was measured according to Maitra and Lobo (1971) and glucose-6-phosphate dehydrogenase (Glc6PDH) activity according to Kuby and Noltman (1966). NADPH-specific glutamate dehydrogenase (NADPH-GluDH) and NAD-specific glutamate dehydrogenase (NAD-GluDH) activities were assayed by using the methods of Doherty (1970) and Corman and Inamdar (1970), respectively, except that both enzyme activities were assayed in 50 mM imidazole pH 7.5, containing 10 mM $MgCl_2$, 100 mM KCl and 0.1 mM EDTA. Protein was determined as described by Zamenhof (1957) using bovine serum albumin as a standard.

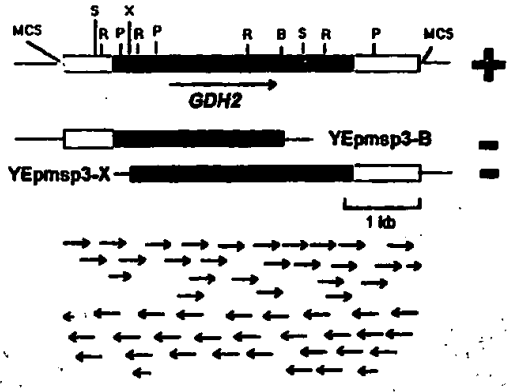

Fig. 1. Restriction map of YEpMSP3 and derivatives and the sequencing strategy. The boxes indicate yeast sequences and the lines indicate YEplac181 vector sequences. The black box indicates the open reading frame of *GDH2*. Suppression results for the deletion derivatives of YEpMSP3 are indicated at the right side: (+), suppression positive; (−) suppression negative. Little arrows mark the direction and length of the sequencing reactions. Abbreviations: B, *Bam*HI; P, *Pst*I; R, *Eco*RI; S, *Sal*I; X, *Xho*I; MCS, multiple cloning site.

Determination of metabolites

Preparation of metabolite extracts was performed essentially as described by Boles and Zimmermann (1993a) and Boles et al. (1993). Metabolite concentrations were determined according to Bergmeyer (1974).

RESULTS

Isolation of multi-copy suppressors

A genomic library in the 2μ-based multi-copy plasmid YEplac 181 (Gietz and Sugino, 1988) was prepared from a partial *Sau*3A digest of DNA of *pgi1* deletion strain EBY23 (Boles and Zimmermann, 1993a) and transformed into this strain. Transformed cells were first plated on a leucine-free medium to select for plasmid uptake and supplemented with 2% fructose and 0.1% glucose to allow all transformants to grow. More than 6000 transformant colonies grew up within four days at 28°C and were replicated onto the same basic medium supplemented with 0.1% glucose as the only carbon source. The replicas of 37 colonies had grown within two weeks. Three of these transformants lost the ability to grow on a medium with 0.1% glucose after 10–15 cell divisions on a nonselective yeast/peptone medium with 2% fructose and 0.1% glucose. This indicated that growth on glucose as the sole carbon source was due to the presence of a multi-copy plasmid-borne suppressor gene. The plasmid in the fastest growing transformant was called YEpMSP3 (for multi-copy suppressor of *pgi1* mutants to grow on glucose).

Characterization of YEpMSP3

Plasmid DNA was isolated from transformant MSP3 and characterized by restriction enzyme analysis (Fig. 1). The complete 4.8-kb insert of YEpMSP3 was recloned into vector YEplac112 (Gietz and Sugino, 1988) with a tryptophan marker to allow for selection of the plasmid in different tryptophan auxotrophic strains. The resulting plasmid YEpMSP3-T was transformed into other glycolysis mutant strains with deletions in the genes coding for pyruvate kinase

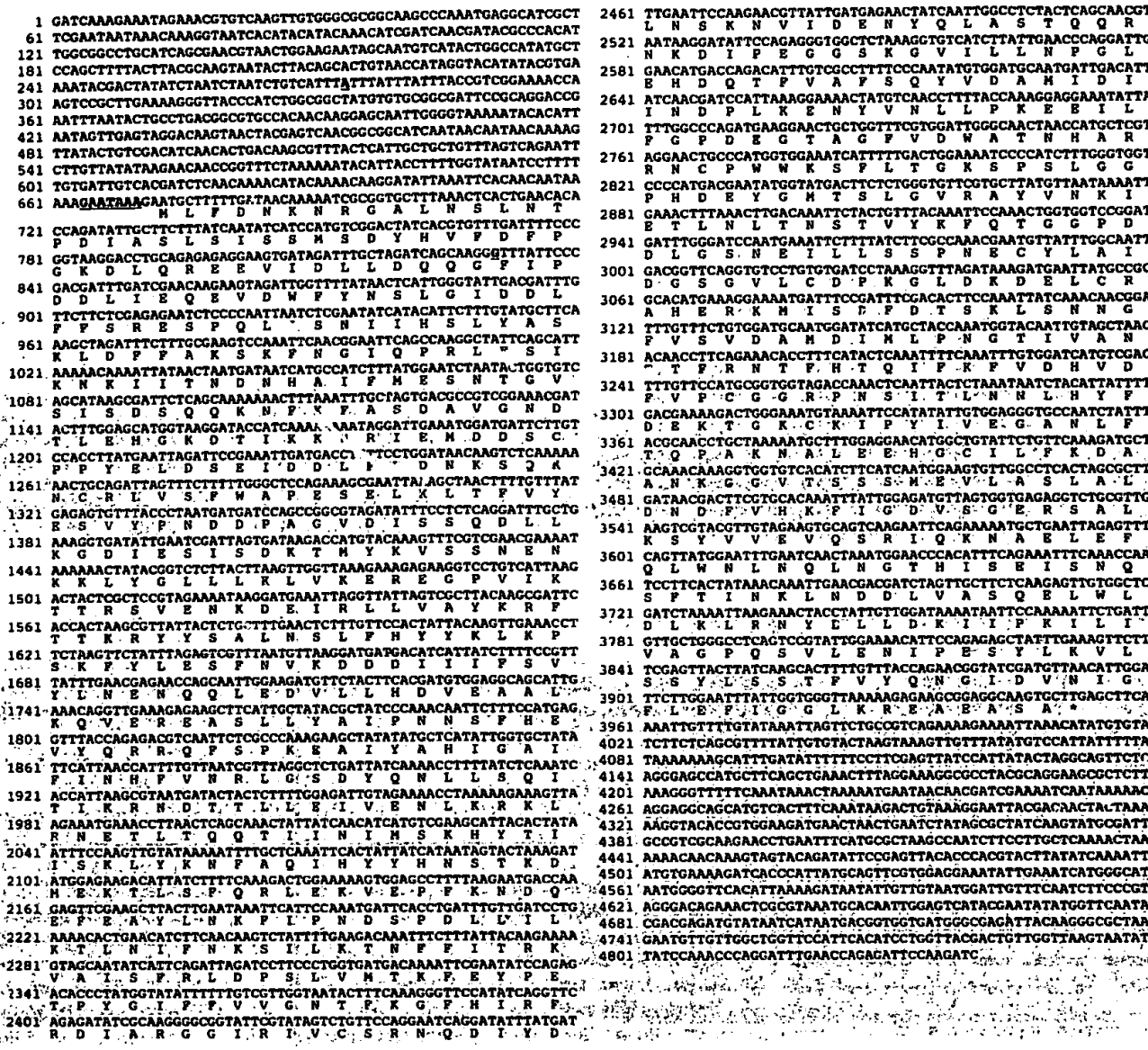

Fig. 2. Sequence of the complete insert of plasmid YEpMSP3 containing the *GDH2* gene. The predicted protein sequence produced by the *GDH2* gene, coding for the NAD-dependent glutamate dehydrogenase, is depicted below the nucleotide sequence. Numbers indicate the positions of nucleic acid residues. Differences to the sequence reported by Miller and Magasanik (1991) are printed in bold and are underlined.

(EBY55), phosphoglycerate mutase (EBY66), phosphoglycerate kinase (EBY71), triosephosphate isomerase (EBY88), fructose bisphosphate aldolase (EBY8) or phosphofructokinase (EBY812) which cannot grow on a glucose medium (Clifton et al., 1978; Ciriacy and Breitenbach, 1979; Boles and Zimmermann, 1993b). However, the ability to grow on glucose was only restored in the *pgi1* deletion strains EBY23 and EBY. UTL-23.

The nucleotide sequence of the complete 4.8-kb DNA insert was determined by sequencing of both strands (Figs 1, 2). A single complete and open reading frame from position 673 bp to 3948 bp of the insert was identified with a predicted coding capacity of 1092 amino acids of a protein of 124 kDa with a codon bias of 0.21 (Bennetzen and Hall, 1982). Suppression of the *pgi1* deletion is due to the open reading frame (ORF) and not to adjacent sequences, because deletions extending into the ORF from either direction abolished *pgi1* suppression (Fig. 1).

The nucleotide and the predicted protein sequence were compared to the databases. The DNA sequence from 2689 bp to 3607 bp still within the open reading frame was found to have about 60% similarity to a partial cDNA sequence of the *Neurospora crassa* NAD-specific glutamate dehydrogenase (GluDH) (Vierula and Kapoor, 1989). Also, at the amino acid sequence level a significant similarity was found to the 1026-amino-acid sequence of the *N. crassa* NAD-GluDH (Austen et al., 1977; Haberland and Smith, 1980) (31% identity in the amino-terminal and 54% identity in the carboxy-terminal half of the proteins). On the other hand, the *GDH2* gene of *S. cerevisiae* coding for the NAD-GluDH had been cloned and mapped by restriction enzyme analysis (Miller and Magasanik, 1990). The restriction enzyme patterns identified an

Table 1. Enzyme activities of NAD- and NADPH-dependent glutamate dehydrogenases in different strains after growth on synthetic complete media (without leucine or uracil) with ammonia as a source of nitrogen, and 2% fructose and 0.1% glucose as the carbon sources. WT = wild-type (ENY. WA-1B); *pgi1Δ*= EBY22; *pgi1Δ ure2Δ*= EBY229; YEplac181 and YEplac195 = cloning vectors; YEpMSP3 = YEplac181 with *GDH2*; YEpGDH1 = pCYG4 of Nagasu and Hall (1985) (YEp13 with *GDH1*); YEpGDH1-U = YEplac195 with *GDH1* of Moye et al. (1985).

Strain	Specific activity of	
	NAD-GluDH	NADPH-GluDH
	mU/mg protein	
WT YEplac181	9.8	236.1
pgi1Δ YEplac181	17.2	271.2
pgi1Δ YEpMSP3	4468.0	306.0
pgi1Δ YEpGDH1	19.6	3099.0
pgi1Δ ure2Δ YEplac195	340.1	158.7
pgi1Δ ure2Δ YEpGDH1-U	289.2	670.2

overlap between the *GDH2* gene and the DNA insert of YEpMSP3 which covers the complete ORF. Miller and Magasanik (1991) reported the sequence of 1300 bp of the 5′-upstream regulatory region and 239 bp of the coding region of *GDH2*. The last part of this sequence overlaps with the first 911 bp of the DNA insert of YEpMSP3 and shows 99.0% identity unequivocally identifying the suppressing gene as the *GDH2* gene of *S. cerevisiae*. Southern blot analysis confirmed that the complete insert of YEpMSP3 is an authentic fragment of yeast DNA and no rearrangements had occurred during cloning of the yeast gene or subsequent propagation of the plasmid (data not shown). We mapped the *GDH2* gene by genomic Southern blot of yeast chromosomes separated by pulse-field electrophoresis. A probe containing the complete 4.8-kb insert of YEpMSP3 cloned into the pBluescript II SK+ vector hybridized strongly only to chromosome number XII (data not shown).

Characterization of the transformants

The *pgi1* deletion strain EBY22 with plasmid YEpMSP3 (with *GDH2*) showed a 260-fold over-expression of NAD-GluDH (Table 1) as compared to strain EBY22 with plasmid YEplac181 (without the *GDH2* gene).

The *pgi1* deletion strain transformed with YEpMSP3 grew on complex yeast/peptone and the mineral salts synthetic complete and synthetic minimal media supplemented with 0.1% glucose (Fig. 3), 1% glucose, and 2% fructose in combination with 2% glucose. Doubling times in a liquid mineral salts synthetic minimal medium supplemented with 0.1% glucose were 2.5 h for wild type and 6–10 h for the transformed *pgi1* deletion strain; however, no phosphoglucose isomerase activity was detectable in crude extracts, nor did transformant strains grow on a mineral salts medium with only fructose.

The respiratory inhibitor antimycin A blocked the growth of the YEpMSP3 transformants on glucose-containing media. Therefore, suppressor activity depended on a functional respiratory system as already noted for the chromosomal suppressor mutants of Aguilera (1987) and Gamo et al. (1993).

Proposal of a model for suppression of *pgi1*

The physiological properties of the *pgi1* deletion strain with the *GDH2* over-expressed on a multi-copy vector can be explained by the following model (Fig. 4). In the first moments after addition of glucose to *pgi1* deletion cells, glucose is oxidatively degraded to ribulose 5-phosphate with the concomitant reduction of NADP to NADPH in the reactions catalyzed by glucose-6-*P* and 6-phosphogluconate dehydrogenases. This causes a rapid decrease in the level of NADP which cannot be regenerated from NADPH rapidly enough so that glucose 6-phosphate can no longer be degraded. Over-expression of the catabolic NAD-GluDH causes an elevated oxidative deamination of glutamate to 2-oxoglutarate with a simultaneous reduction of NAD to NADH. Normally, *pgi1* mutant cells exhibit very low NAD-GluDH levels in a glucose/ammonia medium (Table 1). Reductive amination of 2-oxoglutarate to glutamate by the NADPH-GluDH generates

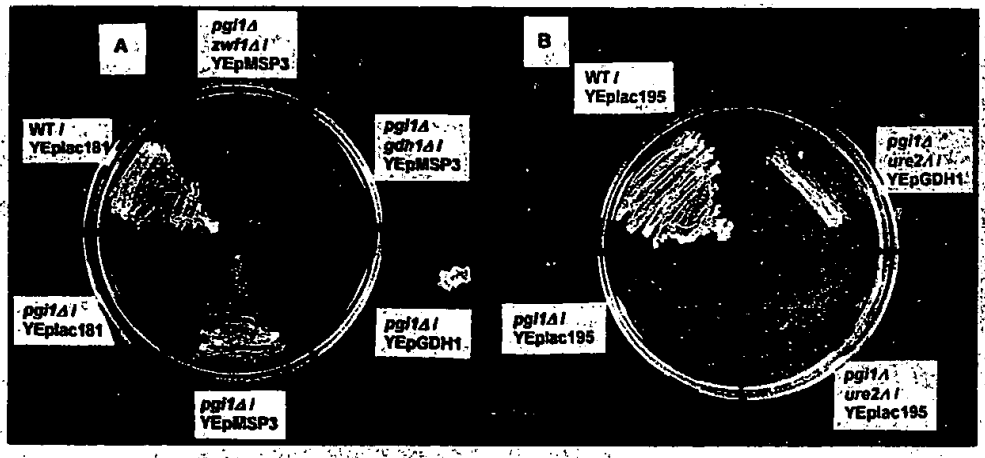

Fig. 3. Growth properties of different mutant strains. Cells were streaked out on synthetic complete media supplemented with 0.1% glucose as the carbon source and lacking leucine (A) or uracil (B). Agar plates were incubated at 28°C for 3 days (A) or 7 days (B). Strains: WT = wild-type ENY. WA-1B; *pgi1Δ* = EBY22; *pgi1Δ /gdh1Δ* = EBY227; *pgi1Δ /zwf1Δ* = EBY228; *pgi1Δ /ure2Δ* = EBY229. The strains were transformed with different plasmids: YEplac181 and YEplac195 = cloning vectors; YEpMSP3 = YEplac181 with *GDH2*; YEpGDH1 (A) = pCYG4 of Nagasu and Hall (1985) (YEp13 with *GDH1*) and YEpGDH1 (B) = YEplac195 with *GDH1* of Moye et al. (1985) (YEpGDH1-U).

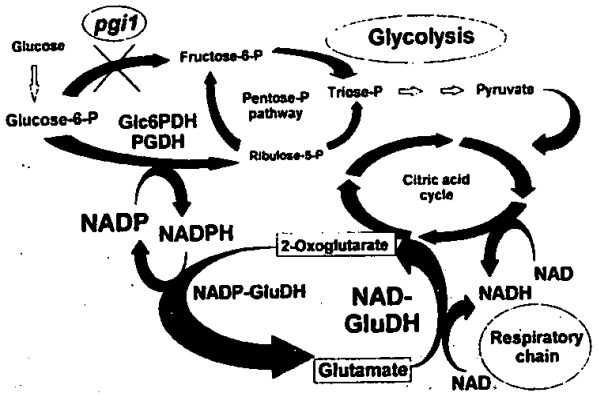

Fig. 4. Scheme of the proposed model for *pgi1* suppression by high-level expression of NAD-GluDH. See text for further information.

NADP required for the catabolism of glucose 6-phosphate to ribulose 5-phosphate. NAD can be regenerated from NADH by the respiratory system whose function is required for the suppression of the *pgi1* deletion defect. Thus, over-expression of *GDH2* creates a cyclic transhydrogenase system which oxidizes NADPH to NADP under generation of NADH.

Additional experimental support of the model

Determination of metabolites confirmed the assumptions (Table 2). After incubation with 0.1% glucose, the levels of nearly all glycolytic metabolites, 6-phosphogluconate, sedoheptulose-7-*P*, glutamate, 2-oxoglutarate and ATP were elevated in a *pgi1* mutant if transformed with YEpMSP3. However, in most cases they were still lower as in the wild-type cells. Interestingly, NADP levels were below the level of detection in the *pgi1* deletion mutant, while over-expression of *GDH2* increased the level of NADP to about half of wild-type levels. Glucose 6-phosphate concentrations were lower in the transformants but still very high as compared to the wild-type cells.

Interruption of the glutamate dehydrogenase cycle by deleting the *GDH1* gene coding for the NADPH-GluDH in a *GDH2* over-expressing *pgi1* mutant strain blocked growth on a pure glucose medium (Fig. 3) when replica-plated from permissive synthetic complete medium without leucine and with 2% fructose and 0.01% glucose. *gdh1* deletion mutant cells exhibited only slightly reduced growth rates in a wild-type background. Growth was also slightly reduced in a *pgi1* mutant background with 2% fructose plus very low amounts of glucose (data not shown) but the double deletion mutant was still more sensitive to low amounts of glucose and inhibited by glucose concentrations higher than 0.06% in combination with fructose whereas the *pgi1* single deletion mutant under the same conditions was only inhibited by glucose concentrations higher than 0.15%.

The model proposes that over-expression of *GDH2* only relieves the inhibitory effects of the glucose and does not restore a conversion of the hexose 6-phosphates by other reactions but only provides for a transhydrogenase system. The structural gene for glucose 6-phosphate dehydrogenase *ZWF1* can be deleted without any obvious metabolic defects in glucose catabolism (Nogae and Johnston, 1990; Thomas et al., 1991). A double deletion mutant *pgi1/zwf1* (strain EBY228) was inhibited on a fructose medium by glucose concentrations higher than 0.05%. Furthermore, over-expression of *GDH2* on plasmid YEpMSP3 did not restore growth on a pure glucose medium (Fig. 3).

The *URE2* gene product is a negative regulator of *GDH2* transcription (Drillien and Lacroute, 1972; Drillien et al.,

Table 2. Concentrations of metabolites. Cells of strains ENY. WA-1B (wild-type) and EBY22 (*pgi1Δ*) were grown overnight in a yeast/peptone medium with 2% fructose and 0.1% glucose, washed once with water and shifted into yeast/peptone medium supplemented with 0.1% glucose 2 h before preparation of extracts. Cells of strain EBY22 carrying the multi-copy plasmid YEpMSP3 with the *GDH2* gene (*pgi1Δ*/YEpMSP3) were first grown overnight in yeast/peptone medium with 0.1% glucose, washed once with water and then shifted into a yeast/peptone/0.1% glucose medium 2 h before preparation of the extract.

Metabolite	Concentration in		
	WT (ENY. WA-1B) 0.1% glucose	*pgi1Δ*/YEpMSP3 0.1% glucose	*pgi1Δ* (EBY22) 0.1% glucose
	nmol/mg dry mass		
Glucose-6-*P*	2.07	58.60	76.20
Gluconate-6-*P*	0.16	1.09	0.27
Sedoheptulose-7-*P*	0.52	0.39	< 0.10
Fructose-6-*P*	0.43	< 0.10	< 0.10
Fructose-1,6-*P*	4.99	0.81	0.25
Triose-*P*	0.73	0.50	0.13
Glycerate-3-*P*	1.34	1.01	0.38
Glycerate-2-*P*	0.18	0.33	0.21
Phospho*enol*pyruvate	0.29	0.66	0.26
Pyruvate	0.74	0.69	0.36
2-Oxoglutarate	1.43	1.28	0.83
Glutamate	74.27	31.16	21.52
ATP	5.30	3.56	0.87
NAD	3.69	4.96	3.91
NADH	3.18	4.09	3.38
NADP	0.25	0.14	< 0.10
NADPH	0.70	0.98	0.77

1973; Coschigano and Magasanik, 1991). In *pgi1/ure2* double deletion mutants NAD-GluDH activity was strongly elevated but, at the same time, NADPH-GluDH activity was reduced to about one half of the value of the *pgi1* single deletion mutant (Table 1). *pgi1/ure2* double deletion mutants could not grow on a pure glucose medium (Fig. 3) but increasing the level of the NADPH-dependent GluDH by transformation with plasmid YEpGDH1-U (Table 1) enabled the double mutant cells to grow very slowly on 0.1% glucose (Fig. 3). On the other hand, a more than tenfold elevated NADPH-GluDH level in the *pgi1* single deletion mutant EBY22 by transformation with plasmid pCYG4 carrying *GDH1* (Nagasu and Hall, 1985) did not at all restore growth on pure glucose medium (Table 1, Fig. 3).

The model suggested that conditions causing an increased oxidation of NADPH will also suppress the phosphoglucose isomerase defect. Oxidizing agents are known to reduce the level of NADPH, presumably via the reaction of glutathione reductase (Kosower and Kosower, 1969; see Nogae and Johnston, 1990). For the experimental generation of oxidative stress, H_2O_2 or electron-transferring chemicals, such as menadione, which transfer electrons from a cellular donor to molecular oxygen (Hassan and Fridovich, 1979) can be used (Schnell et al., 1992). Actually, addition of H_2O_2 (not shown) or menadione promoted growth of *pgi1* mutant cells on a pure glucose medium (Fig. 5). Furthermore, menadione could not promote growth on glucose of a *zwf1/pgi1* double deletion strain (Fig. 5).

Fig. 5. Restoration of growth of *pgi1* mutant cells by menadione. Growth on yeast/peptone supplemented with 0.2% glucose of (A) wild-type (WT) strain (ENY. WA-1B) with 5 µl 30% menadione (M) as a control; (B) the *pgi1* mutant (strain EBY22) with 5 µl 30% menadione; (C) the *pgi1*-mutant (strain EBY22) with 5 µl H_2O; (D) the *pgi1/zwf1* double deletion mutant (strain EBY228) with 5 µl 30% menadione. Cells were grown first in yeast/peptone/2% fructose + 0.01% glucose liquid media and plated on to the agar plates with identical cell densities. The agar plates were incubated for 4 days at 28°C.

DISCUSSION

Efficient recycling of co-substrates is important for metabolic fluxes. In glycolysis, NAD is the co-substrate in the glyceraldehyde-3-phosphate dehydrogenase reaction and the generated NADH has to be re-oxidized in the alcohol dehydrogenase reaction. A total lack of alcohol dehydrogenase (Ciriacy, 1975) and pyruvate decarboxylase (Schmitt and Zimmermann, 1982; Hohmann and Cederberg, 1990) in *S. cerevisiae* blocks glycolysis. An unexpected result was that glycolysis in *S. cerevisiae* is also blocked in mutants lacking trehalose synthase. This was explained by Hohmann et al. (1993) who suggested that trehalose synthesis functions as a 'metabolic buffer system' to recycle the phosphate residues rapidly bound in glucose 6-phosphate after addition of glucose back to inorganic phosphate which is needed for the glyceraldehyde-3-phosphate dehydrogenase reaction.

Mutants with reduced phosphoglucose isomerase activities accumulate high concentrations of glucose 6-phosphate when supplied with glucose, as already noted by Maitra (1971) who interpreted the inhibitory effect of glucose as a toxicity of glucose 6-phosphate because a limited amount of growth should be possible by the 'direct oxidation' of glucose 6-phosphate via the pentose phosphate pathway. Our data suggest that it is the rapid consumption of NADP and an insufficient rate of regeneration of NADP from NADPH which prevents the 'direct oxidation' from operating. However, establishing a transhydrogenase system by a massive over-expression of the NAD-dependent glutamate dehydrogenase or oxidative stress induced by H_2O_2 or menadione which causes a strong oxidation of NADPH suppress the inability of phosphoglucose isomerase mutant to grow on glucose. Both conditions establish a new example for a metabolic regeneration system.

NAD-GluDH serves a catabolic function catalyzing the NAD-dependent oxidative deamination of glutamate to 2-oxoglutarate and ammonium ion (reviewed by Magasanik, 1992). The gene *GDH2* coding for NAD-GluDH was cloned previously and partially sequenced by Miller and Magasanik (1990, 1991). Expression of *GDH2* is subjected to nitrogen and carbon regulation (Courchesne and Magasanik, 1988; Coschigano and Magasanik, 1991; Minehart and Magasanik, 1991; Coschigano et al., 1991). NADPH-GluDH is the key enzyme of *S. cerevisiae* in the anabolic conversion of ammonia into an organic form by a reductive amination of 2-oxoglutarate to glutamate using NADPH as the cofactor (reviewed by Cooper, 1982). The gene *GDH1* coding for NADPH-GluDH had been cloned and sequenced by Moye et al. (1985) and Nagasu and Hall (1985). NADPH-GluDH levels are high and NAD-GluDH levels are low when ammonium ions are supplied as nitrogen source.

The low wild-type level of NAD-GluDH activity was also found in a *pgi1* deletion mutant incubated in a fructose/glucose/ammonia medium (Table 1). It could be increased either by transformation into the cells of a multi-copy plasmid carrying the *GDH2* gene or by deletion of the *URE2* gene which codes for a negative regulator of *GDH2* expression (Coschigano and Magasanik, 1991). The nearly 300-fold elevated level of NAD-GluDH in the transformants is probably due to a deregulation of the gene if present on a

multi-copy plasmid and an elevated copy number of the plasmid due to selective pressure for very high NAD-GluDH activities. In both cases, growth could only be restored in the presence of sufficient NADPH-GluDH activity. The alternative pathway for the net biosynthesis of glutamate from 2-oxoglutarate by glutamine synthetase and glutamate synthase cannot substitute NADPH-GluDH for providing NADP because the glutamate synthase of *S. cerevisiae* uses NADH as its cofactor (Roon et al., 1974). These facts clearly demonstrate the necessity of a substrate cycling between the two glutamate dehydrogenases. NADPH-GluDH is primary responsible for the replenishment of the NADP pool. NAD-GluDH serves only an indirect function of further metabolizing the glutamate which has been generated by NADPH-GluDH. On the other hand, increasing the level of NADPH-GluDH in a *pgi1* single deletion mutant did not at all restore growth on glucose (Fig. 3). This can be explained by two considerations. First, a high level of NADPH-GluDH removes 2-oxoglutarate which is needed as a substrate for oxidative respiration from the citric acid cycle. Growth on pure glucose medium of *pgi1* transformants over-expressing NAD-GluDH was inhibited by antimycin A indicating that respiration is necessary for growth. Second, glutamate accumulates because it cannot be metabolized fast enough by other reactions and the reversible NADPH-GluDH reaction will reach a steady-state equilibrium.

Furthermore, NADPH-GluDH and NAD-GluDH are both cytosolic enzymes (Hollenberg et al., 1970; Perlman and Mahler, 1970). For this reason no NADP shuttle is needed for the metabolic interplay between the pentose phosphate pathway and the glutamate dehydrogenase cycle.

A *pgi1*-deletion mutant is inhibited by glucose concentrations higher than 0.15% on a 2% fructose medium. This type of glucose sensitivity could also be overcome by *pgi1* transformants over-expressing *GDH2*. On the other hand, *pgi1/zwf1* and to a lesser extent *pgi1/gdh1* double deletion mutants were even more sensitive to trace amounts of glucose added together with the fructose than *pgi1* single mutants. Therefore, NADP depletion cannot be the true reason for glucose sensitivity. Our data cannot unequivocally decide whether accumulation of toxic concentrations of glucose 6-phosphate (Maitra, 1971) or depletion of ATP (Ciriacy and Breitenbach, 1979) is the reason for glucose sensitivity but it is interesting to note that *pgi1* mutants over-expressing *GDH2* could grow on glucose with doubling times only about three times higher than wild-type cells although glucose 6-phosphate was present at very high levels (Table 2). Also, the strong accumulation of hexose phosphates generated in the glycolytic and the pentose phosphate pathways and the depletion of ATP and not a depletion of NADP seem to be the reason for the growth deficiencies on glucose of glycolysis mutants which are blocked in the reactions of phosphofructokinase, fructose bisphosphate aldolase or triosephosphate isomerase because in those mutants growth could not be restored by high-level expression of *GDH2*.

Phosphoglucose-isomerase-negative mutants of *E. coli* (Fraenkel and Levisohn, 1967) and of *K. lactis* (Goffrini et al., 1991) are able to grow on glucose medium, although at a reduced rate. Apparently, they utilize glucose primarily by the pentose phosphate pathway. Csonka and Fraenkel (1977) observed that *E. coli* mutants lacking both phosphoglucose isomerase and the membrane ATPase could no longer grow on glucose. They suggested that in a phosphoglucose isomerase mutant the energy-linked transhydrogenase might be used in NADPH oxidation. Lagunas and Gancedo (1973) supposed that a significant transhydrogenation between pyridine nucleotides is not likely to occur in *S. cerevisiae* growing on glucose. This fact could explain the different properties of phosphoglucose isomerase mutants of different species.

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